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Metabolic Engineering of Acetaldehyde Production by *Streptococcus thermophilus*

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The process of acetaldehyde formation by the yogurt bacterium *Streptococcus thermophilus* is described in this paper. Attention was focused on one specific reaction for acetaldehyde formation catalyzed by serine hydroxymethyltransferase (SHMT), encoded by the *glyA* gene. In *S. thermophilus*, SHMT also possesses threonine aldolase (TA) activity, the interconversion of threonine into glycine and acetaldehyde. In this work, several wild-type *S. thermophilus* strains were screened for acetaldehyde production in the presence and absence of L-threonine. Supplementation of the growth medium with L-threonine led to an increase in acetaldehyde production. Furthermore, acetaldehyde formation during fermentation could be correlated to the TA activity of SHMT. To study the physiological role of SHMT, a *glyA* mutant was constructed by gene disruption. Inactivation of *glyA* resulted in a severe reduction in TA activity and complete loss of acetaldehyde formation during fermentation. Subsequently, an *S. thermophilus* strain was constructed in which the *glyA* gene was cloned under the control of a strong promoter (P_{LacA}). When this strain was used for fermentation, an increase in TA activity and in acetaldehyde and folic acid production was observed. These results show that, in *S. thermophilus*, SHMT, displaying TA activity, constitutes the main pathway for acetaldehyde formation under our experimental conditions. These findings can be used to control and improve acetaldehyde production in fermented (dairy) products with *S. thermophilus* as starter culture.

Yogurt is a product obtained through milk fermentation with a specific yogurt starter culture consisting of a mixture of two species of lactic acid bacteria (LAB), *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* (23). The main roles of this mixed starter in the production of yogurt are (i) acidification through the conversion of lactose into lactic acid, (ii) creation of the viscous texture by the production of exopolysaccharides, and (iii) development of the typical yogurt flavor (29).

The typical yogurt flavor is caused by lactic acid, which imparts an acidic and refreshing taste, and a mixture of various carbonyl compounds like acetone, diacetyl, and acetaldehyde, the latter of which is considered the major flavor component (10, 11, 21, 29). The relatively high concentration of acetaldehyde (in the range of 5 to 21 mg/liter) found in yogurt must be due to a low utilization rate of this metabolite since the yogurt bacteria lack the main enzyme for acetaldehyde conversion into ethanol, alcohol dehydrogenase (12).

The production of acetaldehyde by LAB seems to be strain dependent. *L. delbrueckii* subsp. *bulgaricus* has been reported by some authors to be a greater acetaldehyde producer than *S. thermophilus*, whereas other authors have reported the contrary (21, 27). Although it is presently unclear what the major pathway for acetaldehyde production by LAB is, several metabolic pathways have been shown to lead to its formation (5, 24) and it is possible that more than one metabolic pathway operate simultaneously (Fig. 1). During yogurt fermentation, acetaldehyde can be produced directly from lactose metabolism as a result of pyruvate decarboxylation. It can be produced (i) directly via pyruvate decarboxylase or pyruvate oxidase or (ii) indirectly through the formation of the intermediate acetyl coenzyme A by pyruvate dehydrogenase or pyruvate formate lyase. Furthermore, acetaldehyde can be formed by the activity of deoxyriboaldolase, which degrades thymidine into acetaldehyde and glyceraldehyde-3-phosphate. Finally, while several amino acids can be converted into acetaldehyde via pyruvate as a metabolic intermediate, threonine can be directly converted into acetaldehyde and glycine by the activity of threonine aldolase (TA) (29).

In the yogurt bacterium *S. thermophilus*, the only enzyme with TA activity (interconversion of threonine into acetaldehyde and glycine) seems to be the serine hydroxymethyltransferase (SHMT; EC 2.1.2.1). This is an important enzyme involved not only in the formation of glycine and serine but also in the turnover of folate in all organisms (3, 28). However, the role of TA in acetaldehyde formation in mixed yogurt cultures is still not fully understood (32).

The aim of this work was to investigate the role and importance of SHMT in the production of acetaldehyde by *S. thermophilus*. It was observed that the main pathway for acetaldehyde formation in this microorganism is through the activity of SHMT since the inactivation of the encoding *glyA* gene led to the almost complete abolition of TA activity. This result indicates the absence of an alternative pathway for acetaldehyde production and thus establishes the importance of SHMT in

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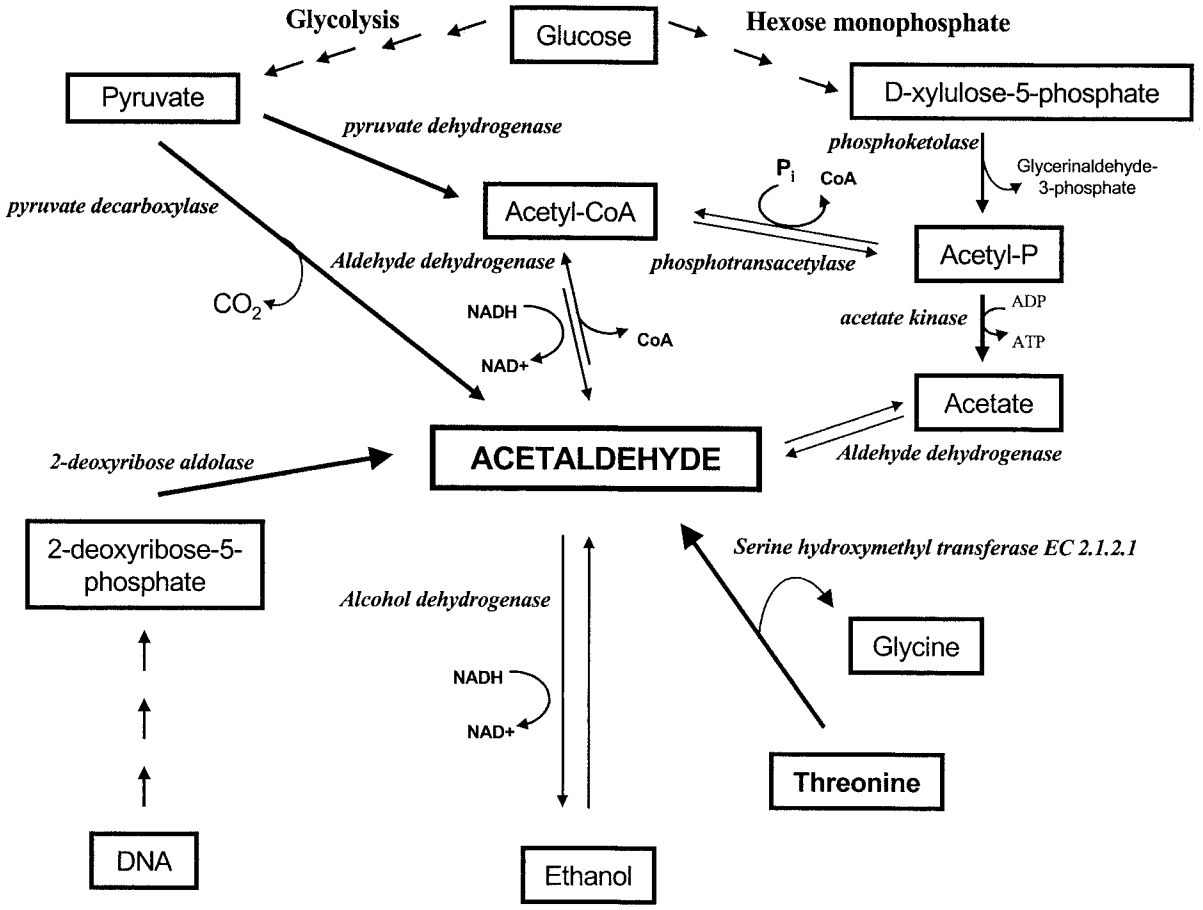

FIG. 1. Overview of the different metabolic pathways in LAB that could lead to acetaldehyde formation. Acetyl-CoA, acetyl coenzyme A.

the production of this key aroma compound. Overexpression of the *glyA* gene showed an increase in TA activity and in acetaldehyde and folic acid formation. These results indicate that, in *S. thermophilus*, SHMT with its TA activity constitutes the main pathway for acetaldehyde formation under our experimental conditions. These findings could be used to control and improve acetaldehyde production in fermented (dairy) products by using *S. thermophilus* as starter culture. They also allow the selection of natural *S. thermophilus* variants with improved flavor-forming characteristics and the improvement of acetaldehyde formation through the metabolic engineering of SHMT.

MATERIALS AND METHODS

Strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. The *S. thermophilus* strains were routinely grown in M17 medium (Brunschwig Chemie, Amsterdam, The Netherlands) supplemented with 1% (wt/vol) lactose (LM17 medium) at 42°C unless stated otherwise. When indicated, the medium was supplemented with 10 or 25 mM L-threonine. *Escherichia coli* was routinely grown in Luria broth-based medium at 37°C with aeration (26). When appropriate, the medium contained erythromycin (150 μg ml^{-1} for *E. coli* or 2.5 μg ml^{-1} for *S. thermophilus*) or ampicillin (50 μg ml^{-1} for *E. coli*). For the growth curve experiments, *S. thermophilus* strains were grown overnight (knockout strains with 2.5 μg of erythromycin ml^{-1}, overexpression strains with 5 μg of chloramphenicol ml^{-1}) and then diluted 100-fold in fresh LM17 medium.

DNA isolation, manipulation, and PCR. Isolation of *E. coli* plasmid DNA and standard recombinant techniques were performed by following established protocols (26). Large-scale isolation of *E. coli* plasmid DNA for nucleotide sequence analysis was performed with the Jetstar plasmid maxprep kit (Genomed GmbH, Bad Oeynhausen, Germany) system following the instructions of the manufacturer. Plasmid and chromosomal DNA from *S. thermophilus* was isolated as described previously for *Lactococcus lactis* (30). Electroporation of *S. thermophilus* was done according to the method of Mollet et al. (18), with the modification that cells were incubated in electroporation buffer at 4°C for 4 h prior to electroporation. Restriction enzymes, *Taq* polymerase, and T4 DNA ligase were used as recommended by the suppliers (R-Biopharm GmbH [formerly Boehringer Mannheim], Darmstadt, Germany; Gibco Invitrogen Corporation, Breda, The Netherlands).

Construction of plasmids for disruption of the *glyA* gene. An internal fragment of the *glyA* gene (505 bp) from *S. thermophilus* NIZOB130 was obtained directly from the genomic DNA by PCR amplification. The degenerated oligonucleotides used (5′-ACNAAYAARTAYGCNGARGG-3′ and 5′-GGNCCNCKNA RNSWYTTRTG-3′, where N is A, C, G, or T; Y is C or T; R is A or G; K is G or T; W is A or T; and S is G or C) were designed based on the sequences of the known GlyA protein from different microorganisms (Fig. 2). The PCR product obtained was cloned in pGEM-T, generating the plasmid pNZ2300. The nucleotide sequence of this cloned fragment was analyzed with an ALFred DNA sequencer (Amersham Pharmacia Biotech, Roosendaal, The Netherlands). Sequencing reactions were performed with an AutoRead sequencing kit and initiated by using Cy5-labeled universal and reverse primers following the instructions of the manufacturer (Amersham Pharmacia Biotech). Sequence data were assembled and analyzed using the CLONE program, version I. Homology searches were performed by using the sequence similarity search program BLAST (22). The CLUSTAL method (8) was used for multiple alignments of

TABLE 1. Bacterial strains and plasmids used and constructed in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
S. thermophilus		
NIZOB103–NIZOB109, NIZOB115–NIZOB118, NIZOB121		Dutch yogurt
NIZOB110 and NIZOB111		Pasteurized milk
NIZOB114		Australian yogurt
NIZOB119		Spray dryer
NIZOB123–NIZOB125		Cheese
NIZOB126 and NIZOB127		Milk
NIZOB128		Cheddar cheese
NIZOB129		Pasteurized milk
NIZOB130, NIZOB1122, and NIZOB1128		Yogurt starter culture
NIZOB131, NIZOB132, and NIZOB133		Italian cheese
NIZOB883–NIZOB886		Thermophilic cheese starter
AO54		17
AO54 harboring pNZ2305		This work
NIZOB130 harboring pNZ2305		This work
NZ2310 (NIZOB130 with the *glyA* gene interrupted)		This work
NZ2311 (AO54 with the *glyA* gene interrupted)		This work
E. coli DH5α		6
Plasmid vectors		
pG$^+$host9		15
pGEM-T		Promega Benelux, Leiden, The Netherlands
pNZ276	Ery^r 4.2 kb with *lacA* promoter	NIZO Food Research
pNZ2300	Amp^r 3.5 kb pGEM-T derived, carrying a 505-bp *glyA* gene fragment	This work
pNZ2310	Ery^r 4.4 kb pG$^+$host9 derived, carrying a 505-bp *glyA* from pNZ2300	This work
pNZ2305	Ery^r 5.5 kb pNZ276 derived, carrying a 1,250-bp *glyA* gene from B130	This work

[a] Amp^r, ampicillin resistant; Ery^r, erythromycin resistant.

sequences. The internal fragment of the *glyA* gene was isolated from plasmid pNZ2300 as an *Apa*I-*Spe*I fragment and then cloned into the vector pG$^+$host9 previously digested with the same enzymes, yielding the plasmid pNZ2310. The vector pG$^+$host9 contains a thermosensitive replicon (15).

Gene disruption. *S. thermophilus* strains NIZOB130 and AO54 were transformed with pNZ2310, and the transformants were selected at 28°C on M17 sucrose (SM17) agar supplemented with erythromycin. In order to facilitate the integration of pNZ2310, overnight cultures grown on SM17 medium at 28°C with erythromycin were diluted 100-fold into fresh medium and then grown at 28°C until early exponential growth phase, when the growth temperature was shifted to 42°C. At 42°C, growth was continued until stationary phase and cells were plated at appropriate dilutions on SM17 agar with erythromycin. These plates were incubated at 42°C; the primary integrates appeared as erythromycin-resistant colonies after 24 to 48 h. In order to confirm the correct integration at the *glyA* locus, Southern hybridization was performed with the 505-bp *glyA* fragment as a probe. The *glyA* mutants of the strains NIZOB130 and AO54 were designated respectively as *S. thermophilus* NZ2310 (NIZOB130Δ*glyA*) and NZ2311 (AO54Δ*glyA*), and they were exclusively grown at 42°C to ensure stable integration of the vector.

Acetaldehyde determination. Acetaldehyde was measured spectrophotometrically by using an acetaldehyde determination kit based on the enzymatic (acetaldehyde dehydrogenase) reduction of NAD to NADH (R-Biopharm GmbH). The acetaldehyde level was determined after 20 h of fermentation at 42°C; the inoculum used was 1.0% of a fresh overnight culture. All assays were performed at least in triplicate.

Enzymatic assay. TA activity of SHMT was monitored with L-threonine as a substrate, and the enzyme activity towards L-threonine was measured as described by Wilkins et al. (33) with some modifications. This method basically measures the threonine-dependent formation of acetaldehyde over time by headspace gas chromatography. Cells were harvested in the exponential growth phase by centrifugation, resuspended in phosphate buffer, and subsequently lysed with a French pressure cell at constant pressure (900 lb/in^2). The cell extracts should be kept on ice from this point until the end of the enzymatic assay. Cell debris was removed by centrifugation (4°C, 10,000 × *g* for 10 min), and the cell extract was used for the enzymatic assay. The reaction was performed at 42°C and terminated by the addition of 600 μl of hydrochloric acid (1 M). The acetaldehyde formed was measured spectrophotometrically (see above), and the specific TA activity was expressed in milliunits (1 mU is 1 nmol of acetaldehyde formed per minute per milligram of protein). Protein concentration was determined using a protein assay kit based on the method of Bradford (2) with bovine serum albumin as the standard (Bio-Rad Laboratories BV, Veenendaal, The Netherlands).

Folic acid determination. The folic acid present in the cells was measured by a microbiological assay with a folate auxotrophic *Lactobacillus casei* strain (7). The method was adjusted for use with microtiter plates as described previously (9, 19).

Construction of a plasmid with the *glyA* gene under the control of the *lacA* promoter. Isolation of the complete coding sequence of the *glyA* gene from *S. thermophilus* NIZOB130 was obtained directly from the genomic DNAs by PCR amplification. The reaction was carried out in a mixture containing 50 μl of 10 mM Tris-HCl (pH 8.55), 25 mM KCl, 5 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, a 0.1 mM concentration of each deoxynucleoside triphosphate, 20 ng of each primer, 1 μg of the genomic DNA, and 0.5 U of Pwo polymerase (Roche Diagnostics Nederland BV, Almere, The Netherlands) at 94°C for 15 s, 55°C for 30 s, and 72°C for 2 min for a total of 30 cycles. The 3′ and 5′ ends of the primer had, based on published *glyA* gene sequences, the sequences 5′-GCGAAAACTGCAGCC TATTAGACCTCAAAG-3′ and 5′-TATCCGCTCGAGTATTAATAGAGTG GGAAAGC-3′, with restriction enzyme sites (*Xho*I and *Pst*I) introduced to allow insertion into the vector pNZ276. The amplified PCR product obtained was digested with *Xho*I and *Pst*I, separated by agarose gel electrophoresis, and purified with the Jetquick gel extraction kit (Genomed GmbH). The amplified DNA fragment (1.2 kb) containing the *glyA* gene sequence starting at the ribosome binding site was inserted downstream of the *lacA* promoter of pNZ276, yielding the plasmid pNZ2305. This construction was stabilized in *E. coli*, and the nucleotide sequence of the amplified *glyA* gene was determined (as described previously) to confirm that no mismatching had occurred during PCR amplification. The plasmid was isolated in large scale by using the Jetstar plasmid maxprep kit (Genomed GmbH), and the pure plasmid was electroporated into the two different strains of *S. thermophilus*, NIZOB130 and AO54.

Overexpression of the *glyA* gene in *S. thermophilus*. A fermentation experiment was conducted with four strains of *S. thermophilus*, the wild-type strains NIZOB130 and AO54 and both of these strains harboring plasmid pNZ2305. They were all fermented in 1 liter of LM17 medium with 5 μg of chloramphenicol/ml when necessary during a 24-h period at 42°C. The decrease of the pH during incubation was continuously monitored with a pH meter microprocessor pMX300 (WTW, Weinheim, Germany). In order to follow the growth of *S. thermophilus*, several samples were taken during the incubation period and serial dilutions were made in sterile physiological salt solutions and deep-plated on

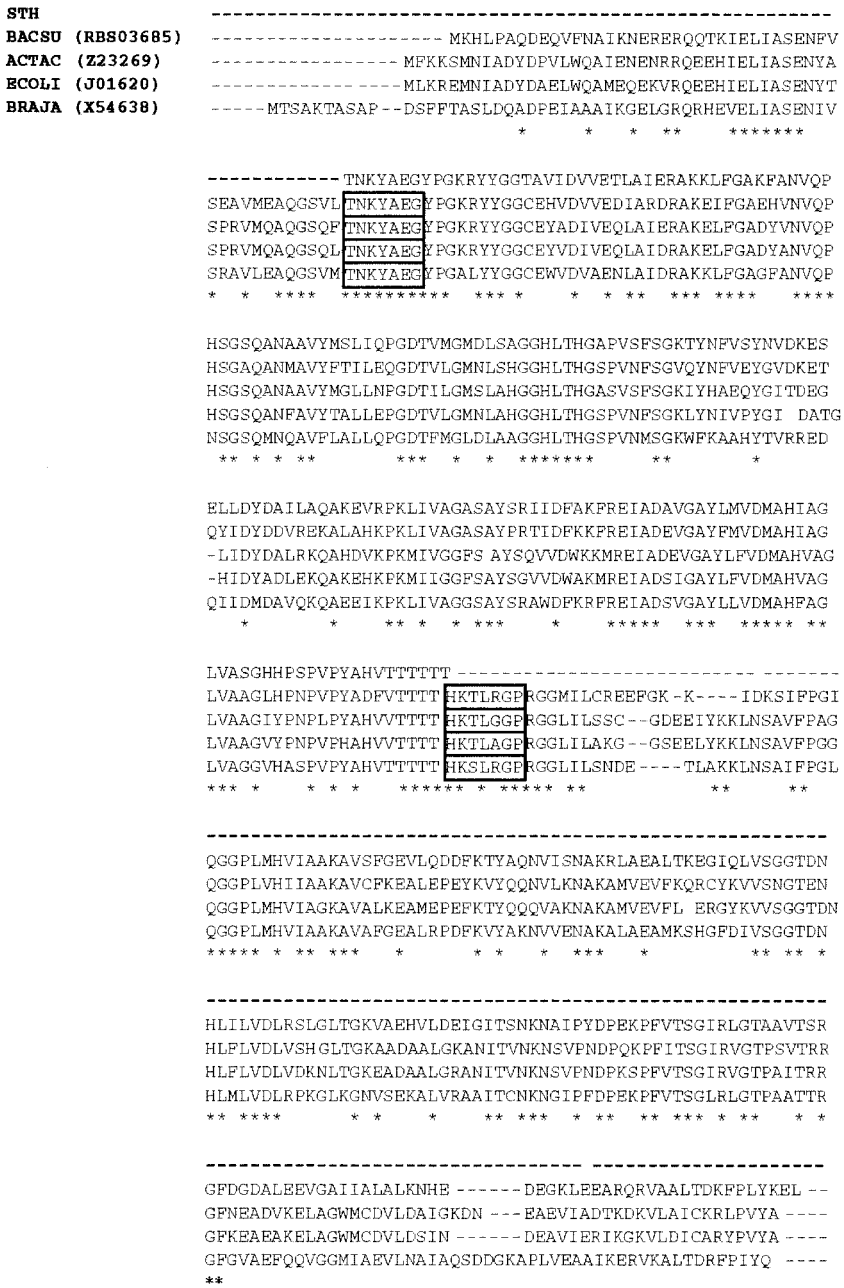

FIG. 2. Multiple alignments of the amino acid sequences encoded by *glyA* genes from *S. thermophilus* NIZOB130 (STH; a partial sequence with 178 amino acids): *A. actinomycetemcomitans* (ACTAC; 420 amino acids), *B. subtilis* (BACSU; 415 amino acids), *B. japonicum* (BRAJA; 432 amino acids), and *E. coli* (ECOLI; 417 amino acids). EMBL database accession numbers are shown in parentheses. Asterisks denote residues conserved among all sequences, and dots represent gaps introduced into a sequence for alignment. The amino acid sequences used for the design of degenerated primers to obtain a part of the *glyA* gene from *S. thermophilus* are boxed (highly conserved regions).

LM17 agar. Plates were incubated for 48 h at 42°C, and microbial count data were expressed as the log of CFU per milliliter. Samples were taken (i) for the TA assay at the end of the exponential growth phase and (ii) to measure folic acid and acetaldehyde production after 24 h of incubation. All experiments and analyses were performed in triplicate.

RESULTS

Acetaldehyde production and TA activity. Acetaldehyde-producing capacity was determined in several wild-type *S. thermophilus* strains. In a first screening experiment, acetaldehyde production was compared for all *S. thermophilus* strains from the NIZO Food Research collection grown in LM17 medium either with (10 or 25 mM) or without L-threonine added (Fig. 3). A clear difference in the amount of acetaldehyde formed could be detected among the different strains. While some strains produced no detectable amounts of acetaldehyde, other strains produced a considerable amount of this product. The level of acetaldehyde formation could be increased in all

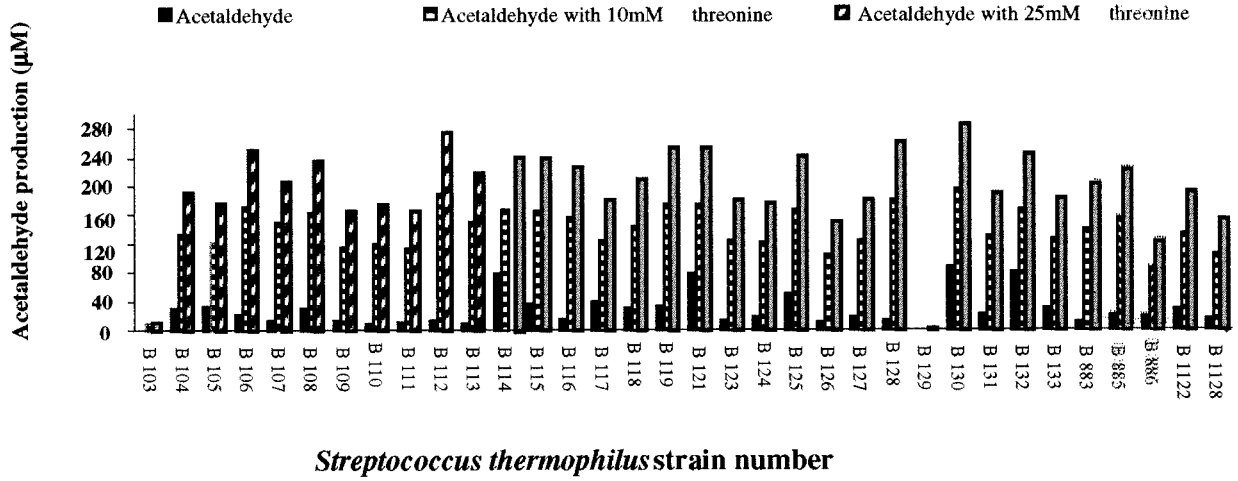

FIG. 3. Screening of *S. thermophilus* strains for acetaldehyde production in LM17 medium in the presence and absence of 10 and 25 mM L-threonine.

strains by adding L-threonine to the culture medium, suggesting the involvement of a TA. The amount of increase appeared to be strain specific and variable. A total of 10 strains, varying in acetaldehyde production between no detectable amounts to relatively high levels (200 μM), were selected for further studies. TA activity and acetaldehyde levels were measured in the selected strains of *S. thermophilus*. The results shown in Fig. 4 reveal that strains producing high levels of acetaldehyde during fermentation also contained high TA activity. This suggests that the TA pathway is responsible for acetaldehyde production in *S. thermophilus* (Fig. 1).

Disruption of the *glyA* gene in *S. thermophilus*. An internal fragment of the *glyA* gene from *S. thermophilus* strains NIZOB130 and AO54 was amplified by PCR with degenerated primers. The resulting PCR product was cloned in pGEM-T, generating the plasmid pNZ2300. Nucleotide sequencing revealed the presence of a single open reading frame of 505 bp that showed high homology with GlyA proteins from other microorganisms. A multiple-amino-acid sequence alignment that includes this internal fragment of GlyA from *S. thermophilus* with known GlyA proteins from various microorganisms is shown in Fig. 2. The sequence of the *S. thermophilus* GlyA fragment showed clear homologies with GlyA from *Bacillus subtilis* (69% identity), *Bradyrhizobium japonicum* (69% identity), *Actinobacillus actinomycetemcomitans* (67% identity), and *E. coli* (66% identity). The highest homology (80% identity) with the *S. thermophilus* GlyA protein was found with a putative GlyA-encoding sequence from another LAB, *L. lactis*, of which the genome sequence was recently published (1; http://www.spock.jouy.inra.fr).

To study the physiological role of the *glyA* gene, a knockout mutant was constructed. The chromosomal *glyA* gene was inactivated by gene disruption with site-specific, temperature-sensitive integration vector pNZ2310 in these two strains of *S. thermophilus*, NIZOB130 and AO54. The *glyA* mutation was confirmed in cells that were able to grow at 42°C under antibiotic selection. One colony of each strain was collected and checked by Southern blot analysis (the hybridization patterns

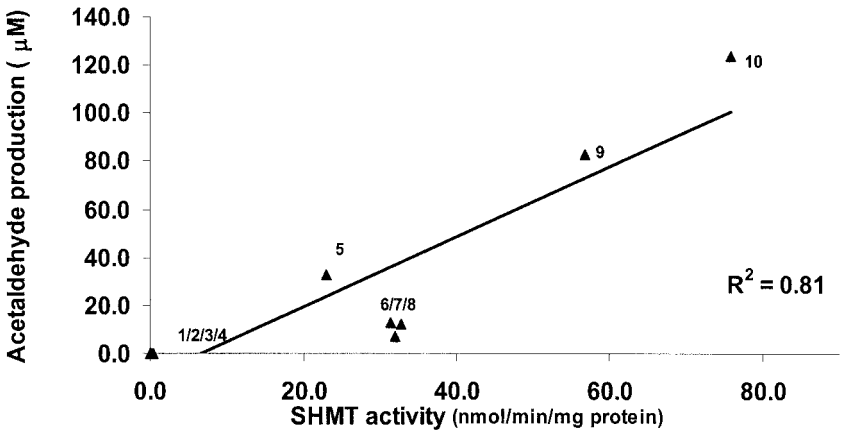

FIG. 4. SHMT activity and acetaldehyde formation during fermentation in LM17 medium at 42°C. The numbers on the graph represent the different strains of *S. thermophilus*: 1, NIZOB103; 2, NIZOB129; 3, NZ2310; 4, NZ2311; 5, NIZOB125; 6, AO54-T; 7, NIZOB1122; 8, AO54; 9, NIZOB130; 10, NIZOB130-T, where T indicates that extra threonine (25 mM) was present during cultivation.

TABLE 2. TA activity, acetaldehyde production, and folate production in the wild-type *S. thermophilus* strains NIZOB130 and AO54, the *glyA*-knockout strains NZ2310 and NZ2311, and the *glyA*-overexpressing mutants harboring pNZ2305

S. thermophilus strain	TA-sp act (mU/mg)	Acetaldehyde production (μM, ppm)	Folate production (mg/liter)
NIZOB130	35 ± 4	4.8 ± 0.5, 109	158 ± 10
NZ2310	2 ± 2	0 ± 0.5, 0	238 ± 20
NIZOB130 + pNZ2305	58 ± 4	8.5 ± 0.5, 193	186 ± 10
AO54	35 ± 6	3.9 ± 0.5, 89	78 ± 5
NZ2311	2 ± 2	0 ± 0.5, 0	135 ± 10
AO54 + pNZ2305	62 ± 6	7.4 ± 0.5, 168	183 ± 15

obtained confirmed the integration of pNZ2310 at the *glyA* locus [data not shown]), and the collected mutants were named NZ2310 and NZ2311. TA activities and acetaldehyde production were determined for both *glyA* mutants in comparison with those of the respective parental strains (Table 2). Inactivation of the *glyA* gene resulted in the almost complete inactivation of TA activity in both strains, indicating that TA activity in *S. thermophilus* resides in SHMT encoded by the *glyA* gene. Furthermore, no detectable amount of acetaldehyde was produced by these strains during fermentation. The disruption of the *glyA* gene resulted in a sixfold reduction of the growth rate relative to those of the parental strains (data not shown). Moreover, the *glyA* mutation resulted in reduced acidification, reflected by a final pH of the culture of 5.2 relative to that of the wild type, which reached a final pH of around 4.5. The large amount of glycine present in LM17 medium (930 mg/liter) seems to preclude glycine limitation as the cause for this reduced growth rate and acidification. This conclusion was supported by the failure to restore the wild-type growth characteristics of the mutants upon the addition of extra glycine to the growth medium. The observed growth defect in the *glyA* mutants could be related to a malfunctioning in folate metabolism, since SHMT is a key enzyme in the regeneration of methyl tetrahydrofolate from tetrahydrofolic acid (3, 27). Interestingly, (intracellular) folate levels were elevated in both *glyA* mutants of *S. thermophilus*, NZ2310 and NZ2311, compared to those found in their parental strains (Table 2).

Overexpression of the *glyA* gene in *S. thermophilus*. In a number of fermentation experiments, the behavior of the four *S. thermophilus* strains NIZOB130, AO54, NIZOB130 harboring pNZ2305, and AO54 harboring pNZ2305—the last two of which overexpress *glyA*—was compared. The acidification and the growth rate of all four strains were nearly identical. Furthermore, the following observations were made (Table 2): (i) an increase in acetaldehyde production of the *glyA*-overexpressing strains exhibited in strain NIZOB130 harboring pNZ2305 (about 82%) and strain AO54 harboring pNZ2305 (about 91%), (ii) an increase in folic acid production exhibited in strain NIZOB130 harboring pNZ2305 (20%) and strain AO54 harboring pNZ2305 (100%), and (iii) an increase in TA activity exhibited in strain NIZOB130 harboring pNZ2305 (61%) and strain AO54 harboring pNZ2305 (70%).

DISCUSSION

In this paper, we have shown that TA is the major enzyme activity involved in acetaldehyde production in the yogurt bacterium *S. thermophilus.* The screening experiment showed that supplementation of the growth medium with L-threonine led to an increase in acetaldehyde production and that the differences in acetaldehyde formation were correlated to the differences in TA activity. The disruption of the *glyA* gene, coding for the SHMT enzyme with TA activity, resulted in the abolition of acetaldehyde production. In addition, by higher production of SHMT in *S. thermophilus*, it was possible to achieve an increased production of acetaldehyde. This provides the ultimate evidence that the previously described process for acetaldehyde formation by *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* through the breakdown of threonine to glycine (12, 13, 16, 28, 32) is a really important source of acetaldehyde production by *S. thermophilus*.

SHMT (EC 2.1.2.1) is a pyridoxal phosphate- and tetrahydrofolic acid-dependent enzyme. TA (EC 4.1.2.5) is also a pyridoxal phosphate-dependent enzyme and catalyzes the interconversion of threonine and glycine plus acetaldehyde (31). The physiological role of TA is generally believed to be in the production of glycine (4). The observation that *L. lactis* strain Z8, which lacks TA activity, requires glycine for growth supports this hypothesis (25).

SHMT is an important enzyme involved in the folate-dependent interconversion of serine and glycine. Interestingly, the *S. thermophilus* strains that showed no detectable acetaldehyde formation during fermentation are the same strains that show low production of folic acid (34). SHMT is the most widespread glycine biosynthetic enzyme among plants, animals, and microorganisms. The conversion of serine to glycine serves as the major source of one-carbon units that are essential for the biosynthesis of purine, thymidylate, and methionine. Although SHMT from rabbit liver and lamb was shown to have TA activity, SHMT in mung beans, in *E. coli*, and in rats had no detectable TA activity. Therefore, the activity of cleaving threonine to glycine and acetaldehyde is not a general property of SHMT (20).

Several TAs (EC 4.1.2.5) encoded by the *itaE* gene have been isolated from different microorganisms. Amino acid sequence comparison of the encoded enzymes with SHMT enzymes encoded by *glyA* show that these different enzymes are not related (20). In order to detect and clone an *itaE*-like gene from *S. thermophilus* strains NIZOB130 and AO54, PCR was carried out with chromosomal DNA by using degenerate primers that were derived from alignments of different *itaE* genes. Although, different PCR conditions were tried, we were not able to amplify the *itaE* gene in the *S. thermophilus* strains. In addition, a BLAST search was done (http://www.biol.ucl.ac.be/gene/genome) in the almost complete genome sequence of *S. thermophilus* and this did not result in the identification of an *itaE*-like gene (Pascal Hols, personal communication). Once we were unable to detect *itaE*-encoded TA, it seemed logical to postulate that SHMT would be the only source of TA activity

in *S. thermophilus*. This hypothesis was tested by disruption of the *glyA* gene.

This disruption in *S. thermophilus* led to (i) a complete loss of detectable TA activity, suggesting that *S. thermophilus* contains no other enzymes with TA activity, and (ii) a significant reduction in the growth rate, which could not be restored by supplementing the medium with glycine. Because SHMT plays a central role in folate and one-carbon-unit metabolism, this growth defect observed is most probably due to a disturbance in the supplying of one-carbon units for the biosynthesis of purines, thymidylate, and methionine (29).

Although some researchers suggest that the physiological role of SHMT may be related to the production of glycine for growth, in this work, the inactivation of the *glyA* gene did not lead to a specific requirement for glycine. Supplementation of LM17 medium with glycine could not restore growth. In *S. thermophilus*, the role of the *glyA* gene must be different from its role in *E. coli.* In *E. coli*, disruption of the *itaE* gene alone did not affect the growth rate while disruption of the *itaE* gene in combination with the *glyA* gene caused a significant decrease in the growth rate. This suggests that TA is not the major source of cellular glycine in the wild type but that it catalyzes an alternative pathway for glycine when SHMT is knocked out (14). In *S. thermophilus*, however, SHMT seems not to be involved in glycine supply, as our growth experiments clearly show.

In conclusion, we have shown that acetaldehyde formation in *S. thermophilus* is catalyzed by a secondary activity (TA) of SHMT, the key enzyme in folate turnover and one-carbon-unit metabolism. This finding enabled us to enhance TA activity and also acetaldehyde and folic acid production by the overexpression of *glyA*, the gene encoding SHMT. Future work is directed to further explore this enzyme activity and to make use of these findings for the improvement of fermented dairy products.

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